

All Three WW Domains of Murine Nedd4 Are Involved in the Regulation of Epithelial Sodium Channels by Intracellular Na⁺*

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The amiloride-sensitive epithelial sodium channel (ENaC) plays a critical role in fluid and electrolyte homeostasis and consists of α , β , and γ subunits. The carboxyl terminus of each ENaC subunit contains a PPxY motif which is necessary for interaction with the WW domains of the ubiquitin-protein ligase, Nedd4. Disruption of this interaction, as in Liddle's syndrome where mutations delete or alter the PY motif of either the β or γ subunits, results in increased ENaC activity. We have recently shown using the whole-cell patch clamp technique that Nedd4 mediates the ubiquitin-dependent down-regulation of Na⁺ channel activity in response to increased intracellular Na⁺. In this paper, we demonstrate that WW domains 2 and 3 bind α -, β -, and γ -ENaC with varying degrees of affinity, whereas WW domain 1 does not bind to any of the subunits. We further show using whole-cell patch clamp techniques that Nedd4-mediated down-regulation of ENaC in mouse mandibular duct cells involves binding of the WW domains of Nedd4 to three distinct sites. We propose that Nedd4-mediated down-regulation of Na⁺ channels involves the binding of WW domains 2 and 3 to the Na⁺ channel and of WW domain 1 to an unknown associated protein.

The amiloride-sensitive epithelial sodium channel (ENaC)¹ plays a critical role in fluid and electrolyte homeostasis and is widely expressed in absorptive epithelia such as the renal collecting duct (1, 2), the colon (1, 2), the lung, (3, 4) and sweat and salivary ducts (1, 2). It consists of α , β , and γ subunits which are thought to assume a tetrameric $\alpha_2\beta\gamma$ structure at the membrane (5, 6). The carboxyl terminus of each ENaC subunit contains a PPxY sequence (the PY motif), which when mutated or deleted in either the β or γ ENaC subunits leads to Liddle's syndrome, an autosomal dominant form of hypertension (7–11). Therefore, mutating just one PY motif from a single subunit of

the tetrameric ENaC complex is sufficient to lead to a disease phenotype. In *in vitro* systems, identical mutations to those that cause Liddle's syndrome, have been shown to increase amiloride-sensitive Na⁺ current (8, 11–15). This increase is believed to result from the presence of increased numbers of active Na⁺ channels in the cell membrane (12, 16–18), although an increase in channel open probability may also contribute (12, 17, 19).

The PY motifs in the carboxyl termini of ENaC subunits are believed to be necessary for interaction with the WW domains of Nedd4 (20), a widely expressed ubiquitin-protein ligase (20–24). Nedd4 is believed to down-regulate Na⁺ channel activity in response to increases in intracellular Na⁺ (17, 25) by ubiquitinating the channel (16, 25, 26), leading to its endocytosis (16, 18) and degradation (16, 26).

The detailed mechanisms by which Nedd4 interacts with Na⁺ channels remain, however, unclear. Nedd4 consists of a ubiquitin-protein ligase domain, multiple WW domains, and a Ca²⁺ and lipid binding domain (20–22). There is now good evidence that Nedd4 is a functional ubiquitin-protein ligase and that it ubiquitinates ENaC (16, 24–26). The Ca²⁺ and lipid binding domain has been shown to mediate Ca²⁺-dependent redistribution of Nedd4 from the cytoplasm to the cell membrane (27), although the importance of this is unclear given that intracellular Ca²⁺ has no role in the control of Na⁺ channels by intracellular Na⁺ (17, 28). Both mouse and rat Nedd4 proteins contain three WW domains, whereas human Nedd4 has an additional WW domain (20, 22). It has previously been reported that WW domains 1, 2, and 3 from rat Nedd4 can bind to the PY motifs of the β and γ ENaC subunits *in vitro* (20). It is not known however whether all, or only a subset, of these three WW domains are required for Nedd4 to regulate Na⁺ channel activity. In this study, we have used a combination of *in vitro* binding assays and whole cell patch clamp analysis to investigate the role of the WW domains of Nedd4 in mediating the feedback inhibition of Na⁺ channels by raised intracellular Na⁺.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The expression construct used to generate protein containing all three WW domains of mouse Nedd4 fused to glutathione S-transferase (GST) has been described previously (25). Single WW domain-GST constructs were generated by amplifying each WW domain individually by PCR using appropriate primers² followed by cloning into the *Bam*HI/*Eco*RI sites of pGEX-2TK (Amersham Pharmacia Biotech). WW domain mutations were produced according to a published protocol (29), PCR amplified, and cloned into the *Bam*HI/*Eco*RI sites of pGEX-2TK. The regions encoding the carboxyl termini of human and mouse α , β , and γ ENaC subunits were PCR amplified² and cloned into either *Bam*HI or *Bam*HI/*Eco*RI sites of pGEX-2TK.

Cloning ENaC from Mouse Mandibular Duct Cells—The regions

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¹ The abbreviations used are: ENaC, epithelial sodium channel; PY motif, PPxY sequence; GST, glutathione S-transferase; NMDG, N-methyl-D-glucamine; PCR, polymerase chain reaction; wt, wild type; FBP, formin binding protein.

² Primer sequences are available upon request.

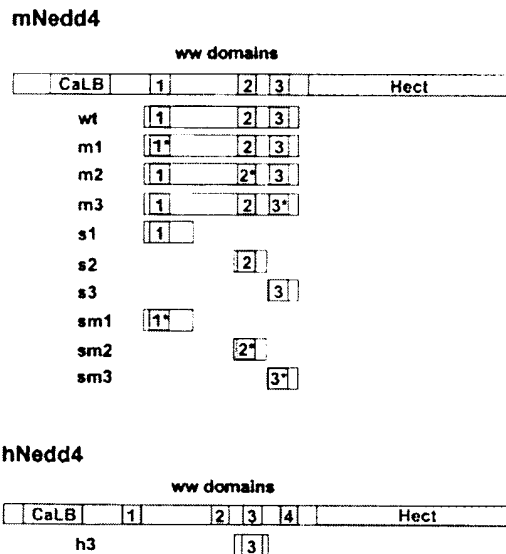


FIG. 1. Schematic representation of the regions of Nedd4 that were fused to GST for protein production. All three WW domains of mouse Nedd4 (*mNedd4*, wt) along with mutants in either WW domain 1 (*m1*), WW domain 2 (*m2*), or WW domain 3 (*m3*) were fused to GST. Single WW domains were also fused to GST in wild-type and mutant forms and are labeled *s1*, *s2*, and *s3* (wild-type WW domains 1, 2, and 3, respectively) and *sm1*, *sm2*, and *sm3* (mutant WW domains 1, 2, and 3, respectively). The third WW domain of human Nedd4 (*hNedd4*, *h3*) was also fused to GST.

encoding the carboxyl termini of the murine α , β , and γ subunits of ENaC were cloned from mouse mandibular duct cells, obtained as described previously (30), by reverse transcriptase PCR. mRNA was reverse transcribed and then PCR amplified using a set of nested primers.² Amplified fragments were cloned into pGEM-T-easy (Promega) and verified by sequencing.

Production of GST Fusion Proteins—Overnight cultures of *Escherichia coli* DH5 α harboring the appropriate GST expression plasmid were diluted 1/50, grown for 2 h at 37 °C, induced with 1 mM isopropyl β -D-thiogalactoside, and grown for an additional 5 h at 37 °C. Bacterial cell pellets were resuspended in phosphate-buffered saline, lysed by sonication, and clarified by centrifugation at 10,000 \times g for 10 min. Glutathione-Sepharose (Amersham Pharmacia Biotech) was incubated with the cleared lysate for 60 min at room temperature, and then the beads were washed three times with phosphate-buffered saline. Fusion protein was eluted with glutathione buffer according to the manufacturer's protocol. Protein concentration was measured using a BCA kit (Pierce).

SDS-Polyacrylamide Gel Electrophoresis and Far-Western Analysis—³²P-labeled protein probes were produced by directly labeling the appropriate GST fusion protein using protein kinase A (New England Biolabs). Glutathione beads containing bound fusion protein were incubated with protein kinase A and [γ -³²P]ATP in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 12 mM MgCl₂, and 1 mM dithiothreitol for 60 min at 4 °C. Beads were washed five times in phosphate-buffered saline, and labeled protein was eluted with glutathione buffer. To prepare WW domain protein filters, approximately 2 μ g of each GST fusion protein was resolved on SDS-polyacrylamide electrophoresis gels and transferred to nitrocellulose membrane (Schleicher & Schüll). Membranes were blocked in Hyb75 (31) and then hybridized with either α , β , or γ ENaC ³²P-labeled protein probes for 4 h at 4 °C in Hyb75. Membranes were washed three times in Hyb75 and exposed to x-ray film.

Whole-cell Patch Clamping—Isolated granular duct cells were prepared by collagenase digestion of mouse mandibular glands from male mice as previously described (30). The standard bath solution (pH 7.4) contained 145 mM NaCl, 5.5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 7.5 mM HEPES, and 10 mM glucose. The standard (0 Na⁺) pipette solution (pH 7.2) contained 150 mM NMDG-glutamate, 1 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, and 10 mM glucose. In the 70 mM Na⁺ pipette solution, Na⁺ was adjusted by substitution of Na⁺ glutamate for NMDG-glutamate. Standard whole-cell patch clamp techniques were used (25, 30). After establishing the whole-cell configura-

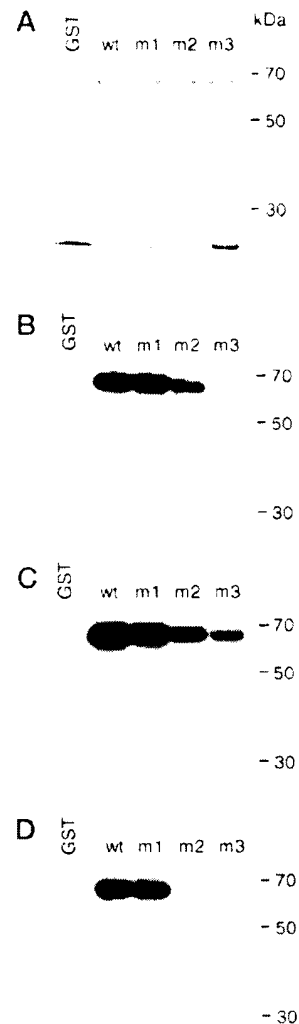


FIG. 2. Varying specificity of α , β , and γ ENaC for Nedd4 WW domains. A, Coomassie Blue stained gel of GST fusion proteins as outlined in Fig. 1 and indicated at top of the gel. Lane 1 contains GST alone and molecular mass markers in kDa are indicated on the right hand side of the gel. B–D, far-Western blots of the above gel probed with ³²P-labeled α , β , or γ ENaC protein probes, respectively.

tion, the bath solution was replaced with one containing 145 mM Na⁺ glutamate, 5 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 1 mM EGTA, and 10 mM glucose (pH 7.4). The amiloride-sensitive current was measured as described previously (25, 30). Results are presented as mean \pm S.E. Statistical significance was assessed using Student's unpaired *t* test. All experiments were performed at 20–22 °C.

RESULTS

Production of GST-WW Fusion Proteins in *E. coli*—To investigate the specificity of the interaction between the WW domains of Nedd4 and the subunits of ENaC, a dual approach involving far-Western blotting and patch clamp analysis was used. To do this, a variety of WW domain proteins fused to GST were produced (Fig. 1). Murine Nedd4 protein contains three WW domains located between the amino-terminal Ca²⁺ and lipid binding domain and carboxyl-terminal ubiquitin-protein ligase domain (22). GST fusion proteins containing all three WW domains of murine Nedd4 with one domain mutated or all three domains in their wild-type configuration were produced as well as wild-type and mutant versions of each individual WW domain (Fig. 1). WW domain mutations were generated by

converting the second conserved Trp to Phe and the conserved Pro to Ala. These mutations were designed to abolish WW domain binding activity without significantly altering the ter-

tiary structure. The human Nedd4 protein contains four WW domains of which domains 1, 2, and 4 correspond to domains 1, 2, and 3, respectively, of the mouse protein (22). These three domains are highly conserved between mouse and human Nedd4 (22) and are thus expected to have similar ligand binding specificity. However, WW domain 3 in human Nedd4 is not found in the mouse protein. We therefore generated a GST fusion protein containing WW domain 3 of the human protein for our binding and patch clamp assays.

α , β , and γ ENaC Subunits Selectively Interact with Nedd4 WW Domains—The PY motifs of α , β , and γ subunits of ENaC have been shown to be required for interaction with Nedd4 WW domains (20), and therefore the intracellular carboxyl terminus of each ENaC subunit containing the PY motif was cloned into the pGEX-2TK vector and used for production of 32 P-labeled protein probes (Fig. 2). Various GST-WW domain fusion proteins were immobilized on nitrocellulose membranes by electrophoresis following electrophoresis on polyacrylamide gels and probed with 32 P-labeled GST-ENaC subunits. As shown in Fig. 2B, the α subunit of ENaC does not bind to the control, GST (lane 1), but binds with equal strength to the wild type (*wt*) protein (lane 2) and the WW domain 1 mutant, *m1* (lane 3), suggesting that it does not bind to WW domain 1. The α subunit of ENaC shows reduced binding ability to mutant WW domain 2 (*m2*) and even less affinity for mutant WW domain 3 (*m3*) which suggests that both WW domains 2 and 3 can bind to the α subunit, although the α subunit has a preference for WW domain 3. The β ENaC subunit has very similar binding characteristics to the α subunit and binds with similar strength to *wt* and *m1* but with reduced affinity to *m2* and *m3* (Fig. 2C). This suggests that the β subunit can bind to WW domains 2 and 3 but not to WW domain 1 and may also have a slight preference for WW domain 3. The γ ENaC subunit bound efficiently to both *wt* and *m1*, once again suggesting that it does not interact with WW domain 1. The γ ENaC subunit bound weakly to *m2* and *m3*, indicating that it has affinity for both WW domains 2 and 3.

To explore the binding specificity of ENaCs further, individual WW domains were fused to GST and used for far-Western analysis. As depicted in Fig. 3B, the α ENaC subunit bound to WW domains 2 and 3 with similar affinity, but not to WW domain 1, confirming the result in Fig. 2B. The α subunit also bound to human WW domain 3, which is not found in mouse Nedd4. As was the case in Fig. 2, the β subunit showed similar binding characteristics to the α subunit by binding to murine WW domains 2 and 3 and human WW domain 3, but not to WW domain 1 (Fig. 3C). The γ ENaC subunit displayed a higher affinity for murine WW domain 2 and human WW domain 3 and reduced affinity for murine WW domain 3, but like the α and β ENaC subunits, showed no affinity for WW domain 1 (Fig. 3D).

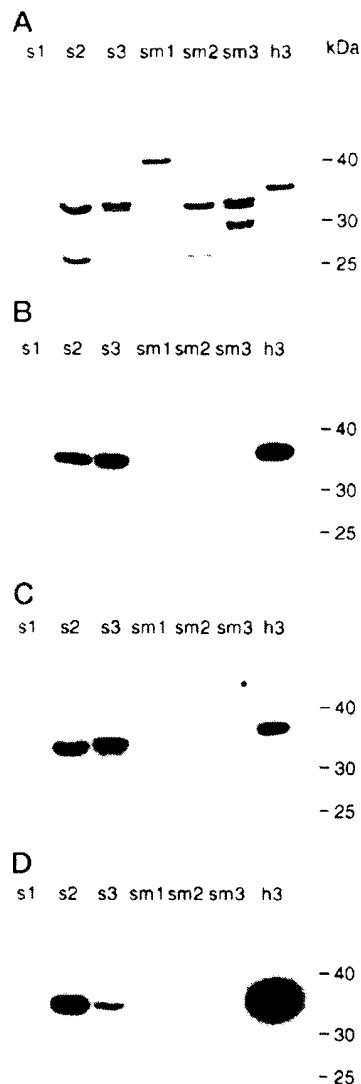


FIG. 3. Specificity of α , β , and γ ENaC for individual Nedd4 WW domains. A, Coomassie Blue stained gel of GST fusion proteins as outlined in Fig. 1 and indicated on top of the gel. Molecular mass markers in kDa are indicated on the right hand side of the gel. B–D, far-Western blots of the above gel probed with 32 P-labeled α , β , or γ ENaC protein probes, respectively.

FIG. 4. A comparison of the carboxyl-terminal amino acid sequences derived from α , β , and γ subunits of mouse and human ENaC. Only the sequences of the 50–60 amino acid residues at the carboxyl termini of the three ENaC subunits are shown. The PPXY motifs present in all three subunits are underlined. The residues identical between the human and mouse proteins are shown in bold. *, termination codon.

ENaC- α	
mouse	FPSRFCHPTSPPPSLPQQGTTPLALTAPPAYATLGPSSASPLDSAVPGSSACAPAMAL*
human	PPSHFCHPMPS...LSLSQGPAPSPALTAPPAYATLGPSPSSGASASSICPLGGP*
ENaC- β	
mouse	HTNFGFQPDITSCRFHGEVYPPDQQLPIPGTFFPNYDSLRLQLDTEMESDSEVFAI*
human	HTNFGFQPDITAPRSPNTGFPYSEQALPIPGTFFPNYDSLRLQLDVIESDSEGDAL*
ENaC- γ	
mouse	PALDITDDDLPTFTSAMRLFPAPFAFVPGTTPPPRYNLTLDLSAFSSQLTDTQTNEF*
human	PALDITDDDLPTNSALHLPPSLGTQVPGTTPPKYNLTLDLRAFAFSSQLTDTQMLDEL*

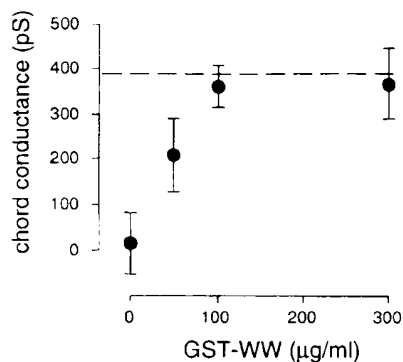


FIG. 5. Dose response curve of inhibition of the Na^+ -dependent feedback pathway by the GST-WW protein. The figure represents the concentration response relationship for the effects of inclusion in the pipette solution of the GST-WW fusion protein on the chord conductance of the amiloride-sensitive current when the pipette contained 70 mM Na^+ . The broken line indicates the mean chord conductance when the 0 Na^+ pipette solution was used.

The WW domain 1 protein (Fig. 1, *s1*) used for binding studies contained an extra 5 kDa at the carboxyl terminus of the WW domain, whereas *s2* and *s3* proteins contained only the minimal WW domain sequence fused to GST (Fig. 1). To examine whether the extra sequence in *s1* altered the binding characteristics of WW domain 1, a shorter construct containing only WW domain 1 was used in far-Western experiments. As was the case with *s1*, this shorter protein did not bind to any of the ENaC subunits (data not shown).

Interactions between Murine ENaC Subunits and Murine Nedd4—The analysis presented in Figs. 2 and 3 was open to objection that interactions between human ENaC carboxyl termini and murine Nedd4 may differ from those we would observe if we were to use murine ENaC carboxyl termini. We therefore, using reverse transcriptase PCR, cloned the coding regions of the carboxyl termini of α , β , and γ subunits of murine ENaC from granular duct cells of mouse mandibular glands. When used in far-Western analyses, GST fusion proteins generated from all three subunits of murine ENaC carboxyl termini gave results similar to those shown in Figs. 2 and 3 (data not shown). This is not surprising given the close homologies evident in and around the PY motifs of human and mouse ENaC subunits (Fig. 4). The PY motifs in the α and β subunits (PPAY and PPNY, respectively) of mouse and human ENaC are identical, whereas in the γ subunit, the sequence differs by a single conservative change (PPRY in mouse compared with PPKY in human) (Fig. 4).

As none of the mouse or human ENaC subunits showed binding affinity for WW domain 1 that was immobilized on a nitrocellulose membrane, we used ^{32}P -labeled WW domain 1 to probe immobilized ENaC-GST fusion proteins. A positive control probe, wt, bound to all mouse and human ENaC subunits, whereas, WW domain 1 did not exhibit any significant binding to any of the subunits (data not shown), further confirming the results shown in Figs. 2 and 3.

All Three WW Domains of Mouse Nedd4 Need to Be Occluded to Inhibit the Na^+ Feedback Loop—As mentioned in the introduction, Nedd4 mediates the feedback inhibition of Na^+ channel activity produced by an increase in intracellular Na^+ . We have previously shown using the whole cell patch clamp technique that Na^+ channel activity of single mouse mandibular granular duct cells can be inhibited by increasing the pipette solution Na^+ to 70 mM (25, 28, 32), a value within the physiological range for cytosolic Na^+ concentration in exocrine tissues (33, 34). This feedback inhibition by raised intracellular Na^+ can be inhibited by inclusion in the pipette solution of: (i) an

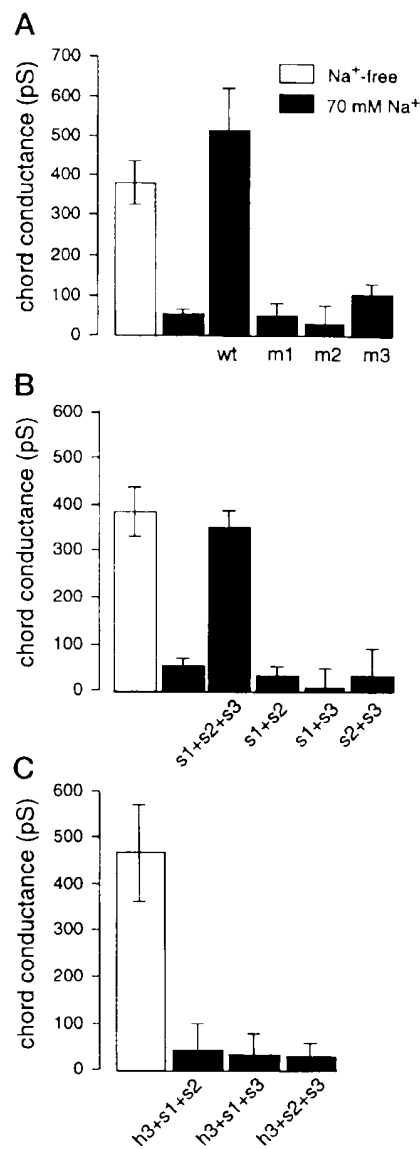


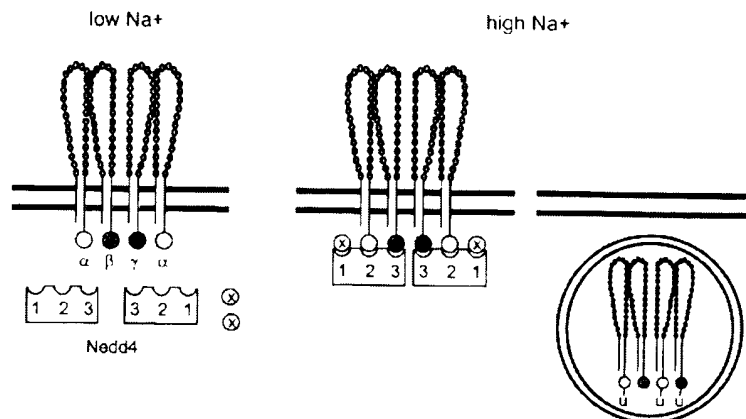
FIG. 6. All three WW domains of Nedd4 are required to inhibit the Na^+ -dependent feedback pathway. A, the effects on the chord conductance of the amiloride-sensitive Na^+ current of the inclusion in the 70 mM pipette solution of the control GST-WW fusion protein (100 $\mu\text{g}/\text{ml}$) and of variants of this fusion protein (100 $\mu\text{g}/\text{ml}$) in which one WW domain has been mutated, leaving the other two intact (*m1*, *m2*, *m3*). B, the effects on the chord conductance of the amiloride-sensitive Na^+ current of the inclusion in the 70 mM pipette solution of various mixtures of fusion proteins containing individual WW domains of murine Nedd4 (*s1*, *s2*, *s3*) in equal concentrations totaling 300 $\mu\text{g}/\text{ml}$. C, the effects on the chord conductance of the amiloride-sensitive Na^+ current of the inclusion in the 70 mM pipette solution of the h3 construct carrying the WW domain 3 of human Nedd4 (100 $\mu\text{g}/\text{ml}$) with pairs of constructs of single WW domains of murine Nedd4 (*s1*, *s2*, and *s3*, 100 $\mu\text{g}/\text{ml}$ each). Details of the constructs used in this figure are given in Fig. 1.

antibody directed against the WW domains of Nedd4,³ (ii) an antibody directed against the ubiquitin-protein ligase domain of Nedd4 (25), or (iii) a GST-Nedd4 WW domain fusion protein that presumably acts as a dominant negative mutant by displacing endogenous Nedd4 from ENaC subunits (25).

We thus decided to use the ability of this GST-WW domain

³ A. Dinudom and D. I. Cook, unpublished data.

Fig. 7. Model for interaction of Nedd4 with ENaC. In response to high intracellular Na^+ , an as yet unidentified factor (X) is activated and binds to Nedd4 via WW domain 1. This serves to recruit Nedd4 to ENaC and/or stabilize the binding of Nedd4 WW domains 2 and 3 to the α , β , and γ subunits of ENaC. We postulate that a stable Nedd4/ENaC complex will comprise one ENaC tetramer, two molecules of Nedd4, and two molecules of factor X. Once stably bound, Nedd4 can ubiquitinate (u) ENaC and stimulate its internalization and/or subsequent degradation.



fusion protein (*wt* in Fig. 1) to block Na^+ feedback inhibition of Na^+ channels as the basis for an investigation of the roles of the individual WW domains of Nedd4 in regulating ENaC activity. We first investigated the concentration-dependence of the ability of the wt protein to block Na^+ feedback inhibition of Na^+ channels in mouse granular duct cells. We found that the inclusion in the pipette solution of 100 $\mu\text{g}/\text{ml}$ GST-*wt*, but not 50 $\mu\text{g}/\text{ml}$ GST-*wt*, was sufficient to overcome feedback inhibition of Na^+ channels (Fig. 5). In subsequent studies we thus used a concentration of 100 $\mu\text{g}/\text{ml}$ as the reference concentration of the GST-*wt* construct.

To test whether all three WW domains of Nedd4 are required to inhibit the Na^+ -dependent feedback pathway, GST fusion proteins containing two intact WW domains and one mutant WW domain (m1, m2, and m3 in Fig. 1) were examined. We found that none of these proteins were able to overcome the inhibition of Na^+ channel activity produced by inclusion of 70 mM Na^+ in the pipette solution (Fig. 6A). These results suggested that each of the three WW domains in murine Nedd4 binds to a distinct, noninterchangeable site and that to prevent Na^+ feedback control of Na^+ channels it is necessary to occlude all three sites. We further tested this interpretation by examining whether the inclusion in the pipette solution of a mixture of individual WW domains of murine Nedd4 (s1, s2, and s3 in Fig. 1, each at 100 $\mu\text{g}/\text{ml}$) could block Na^+ feedback. Indeed, as shown in Fig. 6B, the effect of adding the three individual WW-GST proteins to the pipette solution was similar to the wt WW-GST fusion protein containing the three WW domains. Furthermore, omission of any of these isolated domains from the mixture led to the loss of this blocking effect despite the adjustment of the concentration of each of the two remaining WW domain proteins to 150 $\mu\text{g}/\text{ml}$ to maintain the total concentration of GST-WW domain fusion proteins in the pipette solution at 300 $\mu\text{g}/\text{ml}$ (Fig. 6B). Finally, we examined whether the WW domain unique to human Nedd4 (*h3* in Fig. 1) could replace any of the 3 murine WW domains. We found that it was unable to do so (Fig. 6C). Inclusion of the *h3* domain (100 $\mu\text{g}/\text{ml}$) in the 0 Na^+ pipette solution did not suppress the amiloride-sensitive Na^+ current (data not shown). Thus we concluded that the additional WW domain in human Nedd4 does not substitute for any of the three WW domains that are present in both murine and human Nedd4.

DISCUSSION

This study has shown that to block Na^+ -dependent feedback inhibition of epithelial Na^+ channels, distinct binding sites for all three WW domains of murine Nedd4 must be occluded. It also suggests that, *in vivo*, each of these WW domains binds one of these sites and is unable to bind to the other two. *In vitro*, the WW domains of Nedd4 were found to have varying specificity

for the PY motifs of different ENaC subunits. Murine WW domains 2 and 3, and human WW domain 3 showed varying affinity for all three ENaC subunits, whereas WW domain 1 failed to bind to either of the three ENaC subunits. This suggests that WW domain 1 either prefers a different PY motif to WW domains 2 and 3 or different residues surrounding the PY motif. There is a precedence for WW domains preferring proline-rich motifs other than PPxY; the WW domains of some formin binding proteins (FBPs) prefer a PPLP motif (35, 36), whereas other FBPs prefer a proline-, methionine-, and glycine-rich motif (37). The Nedd4 WW domain 1 is much more homologous to Nedd4 WW domains 2 and 3 than to the WW domains of FBPs, so it is reasonable to expect that WW domain 1 will bind to a PPxY motif containing protein. Staub *et al.* (20) reported that WW domain 1 of rat Nedd4, like WW domains 2 and 3, bound to the PY motif of both the β and γ ENaC subunits. Because mouse and rat Nedd4 WW domain 1 sequences are highly homologous (95% identical), the possibility of different ligand specificities is highly unlikely. Our data show that, similar to mouse, the human Nedd4 WW domain 1 does not interact with either of the three ENaC subunits. We are therefore unsure about the reason for discrepancy between our results and those reported by Staub *et al.* (20). It is worth noting that two other proteins p45/NF-E2 and RNA polymerase II, which interact with Nedd4 through their PY motifs, also do not bind WW domain 1 (38), suggesting that WW domain 1 of Nedd4 may have very restricted ligand-binding specificity.

In light of our binding assays, it may have been reasonable to expect that WW domains 2 and 3 alone would be capable of exerting a dominant negative effect on the Na^+ feedback pathway as measured by patch clamp analysis. This was not the case, however, as an intact WW domain 1 was required, in concert with WW domains 2 and 3, to restore Na^+ channel activity under high Na^+ conditions. We therefore suggest that WW domain 1 must play a role in ENaC regulation that is not dependent on direct binding of an ENaC subunit PY motif. We postulate that WW domain 1 binds to an additional, as yet unidentified, molecule that is activated under high salt conditions, possibly downstream of G_0 . This molecule may recruit Nedd4 to the ENaC complex and/or stabilize the ENaC/Nedd4 complex. We postulate that a stable Nedd4/ENaC complex will comprise one ENaC tetramer, two molecules of Nedd4, and two molecules of the unknown recruitment/stabilization factor (Fig. 7). The possibility also exists that a single molecule of Nedd4 binds to a single ENaC tetramer, leaving two ENaC PY motifs free. Further experiments are required to explore these possibilities. *In vitro*, human WW domain 3 showed affinity for all three subunits of ENaC, comparable with the binding characteristics shown by murine WW domains 2 and 3 (Fig. 3), but it

was unable to complement either of these WW domains or WW domain 1 *in vivo*. This suggests that, although human WW domain 3 recognizes the PY motifs of ENaC subunits *in vitro*, it does not play a role in the Na⁺-dependent feedback inhibition of Na⁺ channels.

The finding that all three WW domains of murine Nedd4 must be occluded to inhibit the Na⁺ feedback loop suggests that more than one WW domain/PY motif contact needs to occur for stable complex formation between Nedd4 and ENaC. This is supported by the molecular understanding of the hypertensive disorder Liddle's syndrome, where mutation in a single ENaC subunit (β or γ) is sufficient to cause a disease phenotype (7–11). As ENaC is a tetramer, presumably three subunits with intact PY motifs will still be present in Liddle's syndrome patients. This would seem insufficient to form a stable complex with Nedd4, however, as hypertension results, presumably as a consequence of lack of negative regulation of ENaC by Nedd4.

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